

Interaction between Transcriptional Activator Protein LAC9 and Negative Regulatory Protein GAL80

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In *Saccharomyces cerevisiae*, transcriptional activation mediated by the GAL4 regulatory protein is repressed in the absence of galactose by the binding of the GAL80 protein, an interaction that requires the carboxy-terminal 28 amino acids of GAL4. The homolog of GAL4 from *Kluyveromyces lactis*, LAC9, activates transcription in *S. cerevisiae* and is highly similar to GAL4 in its carboxyl terminus but is not repressed by wild-type levels of GAL80 protein. Here we show that GAL80 does repress LAC9-activated transcription in *S. cerevisiae* if overproduced. We sought to determine the molecular basis for the difference in the responses of the LAC9 and GAL4 proteins to GAL80. Our results indicate that this difference is due primarily to the fact that under wild-type conditions, the level of LAC9 protein in *S. cerevisiae* is much higher than that of GAL4, which suggests that LAC9 escapes GAL80-mediated repression by titration of GAL80 protein *in vivo*. The difference in response to GAL80 is not due to amino acid sequence differences between the LAC9 and GAL4 carboxyl termini. We discuss the implications of these results for the mechanism of galactose metabolism regulation in *S. cerevisiae* and *K. lactis*.

The *Saccharomyces cerevisiae* GAL4 gene encodes an 881-amino-acid protein required for transcriptional activation of the galactose-melibiose regulon (14, 19, 24). Transcriptional activation is achieved primarily through two separable functions of the GAL4 protein. One function is mediated by the amino-terminal 74 amino acids of GAL4, which bind specific DNA sequences termed UAS_G (GAL upstream activating sequences) located upstream of regulated structural genes (2, 3, 12, 13, 18, 40, 42). The other is a transcriptional activation function executed by the carboxy-terminal 28 amino acids which presumably interact with cellular transcription factor(s) (4, 20, 27). In the absence of the inducer (galactose or a metabolic derivative), GAL4 activity is blocked specifically by the GAL80 negative regulatory protein (9, 32). The inducer abolishes the repressive effects of GAL80 (9). Mutations in the GAL80 gene, termed GAL80^s, encode proteins which repress GAL4 under both inducing and noninducing conditions (10, 31). GAL80^s proteins are thought to possess either an increased affinity for GAL4 protein or a defect in the GAL80 inducer-response function (10, 31).

GAL4 can escape GAL80-mediated repression in two ways. First, some mutations in the GAL4 gene, termed GAL4^c, encode proteins which are not responsive to GAL80 and activate regulon structural genes constitutively (9). Second, the increase of the intracellular dosage of GAL4 by overexpression of the GAL4 gene also causes constitutive structural gene expression (19), presumably through titration of GAL80 by excess GAL4 protein. These findings demonstrate that normal regulation of galactose metabolism in *S. cerevisiae* depends both on specific interactions between the GAL4 and GAL80 proteins and on a proper balance between their intracellular levels.

It has been proposed that GAL80 acts by binding the carboxy-terminal 28 amino acids of GAL4 under noninducing conditions, which would make this region inaccessible to yeast transcription factors (20, 28). The inducer either dissociates GAL80 from GAL4 or changes its conformation in

a way that allows access of the transcription factors to the GAL4 carboxyl terminus. Several experimental observations support this model. First, the GAL4 and GAL80 proteins have been shown to bind each other *in vitro* (26), which demonstrates directly that physical interaction between these two molecules can occur. Second, the six GAL4^c alleles for which sequence data are available all contain either missense or deletion mutations in the region that encodes the carboxy-terminal 28 amino acids of GAL4 (20; J. M. Salmeron, Jr. and S. A. Johnston, submitted for publication), which indicates that alteration of this region can relieve GAL4 of GAL80 repression. Third, overproduction of protein fragments which contain these 28 amino acids, by themselves afunctional either as transcriptional activators or as DNA-binding proteins, causes constitutive activation of the regulon in a GAL4 *S. cerevisiae* strain (21, 28). Presumably, the protein fragments bind and titrate the cellular GAL80 protein, which would relieve wild-type GAL4 from GAL80 repression.

The milk yeast *Kluyveromyces lactis* possesses a galactose metabolism regulon similar to that of *S. cerevisiae* (33). Regulon gene expression is activated by the product of the positive regulatory gene, LAC9 (41). LAC9 also activates expression of the β -galactosidase gene LAC4 (36, 38, 41). The LAC9 protein is thought to activate transcription by binding sites upstream of *K. lactis* structural genes that are homologous to *S. cerevisiae* UAS_G sequences (5, 25, 35). The wild-type *K. lactis* regulatory system is inducible, and mutations in a negative regulatory locus, LAC10, cause constitutive expression of regulon structural genes (8). It is thought that LAC10 encodes a protein which antagonizes LAC9 function in a manner similar to that employed by GAL80 against GAL4.

The LAC9 gene has been cloned and introduced into *gal4* *S. cerevisiae* strains (36, 41). The 865-amino-acid LAC9 protein activates transcription in *S. cerevisiae* but is not measurably repressed by the levels of (chromosomally-encoded) GAL80 protein present in the *S. cerevisiae* strains in which LAC9 activity has been measured (36, 41). The predicted protein sequences of LAC9 and GAL4 are highly

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similar in three regions, present in the amino-terminal, central, and carboxy-terminal portions of the proteins (36). The amino-terminal region of similarity corresponds to the DNA-binding domain of GAL4 (22). The central region lies in an area of unknown function in both proteins. The carboxy-terminal region of similarity corresponds to the 28-amino-acid region of GAL4 which contains both transcriptional activation and GAL80-interactive functions.

The existence of homology between a region of LAC9 and a region of GAL4 identified as a site of interaction with GAL80 led us to look further for evidence of interaction between LAC9 and GAL80, and LAC9 and GAL80^s proteins in *S. cerevisiae*. Here we report that the GAL80 protein, when overproduced, represses LAC9-activated transcription. In addition, normal levels of GAL80^s proteins repress LAC9. Analysis of the LAC9-GAL80 and LAC9-GAL80^s interactions could yield insight into the interaction of GAL4 with GAL80 and GAL80^s proteins. Therefore, we sought to determine whether the difference in the ability of wild-type GAL80 to repress LAC9 and GAL4 arises from specific differences between the LAC9 and GAL4 amino acid sequences, differences in the intracellular levels of LAC9 and GAL4, or both. Our results indicate that the intracellular levels of LAC9 and GAL4, but not the amino acid sequences, are important in determining the difference in the responses of these two proteins to GAL80. These studies underscore the importance of a balance between the levels of positive and negative factors in the proper regulation of gene expression and provide insight into the mechanisms underlying GAL80^s mutations. They also suggest a plausible explanation for the absence of glucose repression of the *K. lactis* regulon.

MATERIALS AND METHODS

Strains. For enzyme assays and electrophoretic mobility shift assays, *S. cerevisiae* strains YJ4 (a *gal4Δ-557 ura3-52 leu2-3,112 GAL80 LAC4::HIS3 MEL1*) and YJ0 (a *gal4Δ-557 gal80Δ-538 ura3-52 leu2-3,112 his3Δ-100 ade2-101 MEL1*) were used. YJ4 is the product of a cross between strains SJ21R (19) and a derivative of YM582 (α *gal4Δ-537 ura3-52 his3Δ-200 ade2-101 lys2-801 tyr1*) in which the *K. lactis* LAC4 gene has been integrated at the *HIS3* locus (36). YJ0 is the product of a cross between strain Sc18 (a *gal80Δ-538 ura3-52 leu2-3,112 his3Δ-200 MEL1*) and YM706 (α *gal4Δ-542 ura3-52 his3Δ-200 ade2-101 lys2-801 trp1Δ-901 tyr1*). Sc18 is the product of a cross between strain SJ21 (19) and YM704 (α *gal80Δ-538 ura3-52 his3Δ-200 ade2-101 lys2-801 trp1Δ-901*). YM582, YM706, and YM704 were gifts from Mark Johnston. Wild-type *K. lactis* Y-1140 (obtained from the U.S. Department of Agriculture) was used for electrophoretic mobility shift assays.

Plasmid manipulations. Standard methods for plasmid manipulation were followed (30) by using enzymes purchased from Promega Biotec, Madison, Wis. Centromeric plasmids that bear GAL80 and GAL80^s genes were constructed as follows. The 3.2-kilobase *HindIII-HindIII* fragment from pGAL80-50 (43; a gift from Rogers Yocum) which contains the GAL80 gene was cloned into the *HindIII* site of YCp50 (18; a gift from Craig Giroux) to form YCpGAL80. The 1.2-kilobase *EcoRI-HindIII* fragment which contains the 3' end of the GAL80 gene was excised from YCpGAL80 and replaced with the corresponding gene fragment from either pHS71, pHS141, and pHS222 (32; gifts from Yasuhisa Nogi) to form YCpGAL80^{s-0}, YCpGAL80^{s-1}, and YCpGAL80^{s-2}, respectively.

To generate yeast centromeric plasmids that contain either the GAL4 gene or the LAC9 gene, the 3.4-kilobase *BamHI-HindIII* fragment from pSJ3 (19), which contains the GAL4 gene, or the 7-kilobase *Sall-Sall* fragment of pJ431 (36), which contains the LAC9 gene, was cloned into YCp50 and pSB32 (a gift from Craig Giroux, in which the *URA3* gene of YCp50 had been replaced with the *LEU2* gene). The resulting plasmids were designated YCp50-GAL4, YCp50-LAC9, pSB32-GAL4, and pSB32-LAC9.

Carboxy-terminal variants of GAL4 and LAC9 were generated as follows. The wild-type GAL4 and LAC9 gene fragments described above were inserted into the multiple cloning site of pUC119 (39). Single-stranded DNAs were prepared from the resulting plasmids (39) and were used as templates for site-directed mutagenesis with an Amersham in vitro mutagenesis kit. Primers used to direct mutagenesis were 15- to 30-mers synthesized by an Applied Biosystems model 380B DNA synthesizer. Variant GAL4 or LAC9 alleles were sequenced (37) by using Sequenase (United States Biochemical Corp., Cleveland, Ohio) to confirm their identity, cloned into YCp50, and transformed into strain YJ4 for assaying in yeast cells.

YEp351 and YEp352 (15) were gifts from Alexander Tzagoloff.

Plasmids were introduced into yeast cells by either the spheroplast (16) or lithium chloride (17) method.

α-Galactosidase enzyme assays. Transformants of strains YJ4 or YJ0 were grown in complete medium, lacking uracil for maintenance of plasmid. Carbon sources were 3% glycerol-2% lactic acid for uninduced cultures and 3% glycerol-2% lactic acid-2% galactose for induced cultures. Assays were performed either with intact cells (23) or with cell extracts (19).

Electrophoretic mobility shift assays. Details concerning the preparation of yeast cellular extracts and performance of electrophoretic mobility shift assays are described elsewhere (J. C. Corton and S. A. Johnston, submitted). The DNA used for the assays reported here consisted of two oligonucleotides, 5'-CTAGGAGCGGGTGACAGCCCTCCGAAT-3' (UAS_G3) and 5'-CTAGATTCGGAGGGCTGTACCCCGC TC-3' (UAS_G4), which had been annealed to form a GAL4 binding site (12) termed UAS_G3-4 and end labeled with [γ -³²P]ATP (30).

For testing the relative dissociation rates of LAC9 and GAL4 from UAS_G3-4 (Fig. 3), 200 μg of either YEp351-GAL4 or YCp50-LAC9 *S. cerevisiae* extract was incubated with 0.8 ng of [³²P]UAS_G3-4 in 120 μl of Buffer A (2) at ambient temperature for 20 min. A 15-μl portion (each 15-μl portion contained 25 μg of protein, 0.1 ng of [³²P]UAS_G3-4, and 0.5 ng of unlabeled UAS_G3-4) was removed and set aside as a control. A 4-μl portion of a Buffer A solution containing 4 ng of unlabeled UAS_G3-4 was added to the remainder of the binding mixture. After 0, 1, 2, 3, 4, 5, and 10 min, a 15-μl portion of the binding mixture was removed from the reaction tube and immediately electrophoresed through a 2.5% NuSieve (FMC Bioproducts, Rockland, Maine)-0.5% low-melting-temperature agarose gel. The control samples were loaded onto the gel 10 min after the bulk of the binding mixture had been exposed to unlabeled UAS_G3-4. The gel was run for 1.5 h at 100 V, fixed in 10% trichloroacetic acid, blotted, dried, and autoradiographed.

RESULTS

LAC9 interacts with GAL80. Our previous work (20, 21) and that of Ma and Ptashne (28) identified the GAL4 car-

TABLE 1. Effect of overexpression of the *GAL80* gene on LAC9-activated transcription

Activator protein and source of <i>GAL80</i> ^a	Mean relative α -galactosidase level (\pm SE) under indicated conditions		R ^b
	Uninduced	Induced	
LAC9			
Chromosome	54.1 \pm 5.3	51.2 \pm 3.6	1.0
YEp13-GAL80	9.2 \pm 1.8	54.4 \pm 11.5	5.9
YEp352-GAL80	8.0 \pm 0.9	54.7 \pm 4.7	6.8
GAL4			
Chromosome	0.3 \pm 0.1	100.0 \pm 6.0 ^c	333.3
YEp13-GAL80	0.3 \pm 0.1	44.1 \pm 6.2	147.0
YEp352-GAL80	0.3 \pm 0.1	19.2 \pm 2.4	64.0
None			
YEp352-GAL80	<0.1	<0.1	

^a Activator proteins encoded by either YCp50-LAC9, pSB32-LAC9, YCp50-GAL4, or pSB32-GAL4. Strains in which the *GAL80* gene is chromosomally borne are transformants of strain YJ4; strains in which the *GAL80* gene is plasmid borne are transformants of strain YJ0.

^b R, Induction ratio for α -galactosidase (induced level to uninduced level).

^c The reference value 100.0 represents an increase in optical density at 400 nm of 0.338/min per 10⁷ cells.

boxyl terminus as a site of interaction with the GAL80 protein. Since high sequence similarity exists between the LAC9 and GAL4 carboxyl termini (36, 41), we reasoned that LAC9 should respond to GAL80, although previous studies failed to detect repression of LAC9-activated transcription by the chromosomally encoded level of GAL80 protein in *S. cerevisiae* (36, 41). Therefore, we investigated further the ability of GAL80 to interact with LAC9. Two different multicopy plasmids, YEp13 (43) and YEp352 (15), bearing the *GAL80* gene were introduced into *S. cerevisiae* strains which contain either the *LAC9* or *GAL4* gene borne on a single-copy plasmid. We tested for the ability of GAL80 to repress transcriptional activation by measuring the amount of α -galactosidase activity (the product of the regulon structural gene *MEL1*) activated by LAC9 in the presence of single and multiple copies of the *GAL80* gene under both noninducing and inducing conditions (Table 1). Whereas the chromosomally encoded level of GAL80 protein was insufficient to repress transcriptional activation mediated by LAC9, overproduction of GAL80 caused an approximately sixfold repression of LAC9-activated transcription under noninducing conditions. We concluded that GAL80 is able to repress the activity of the LAC9 protein.

LAC9 protein interacts with GAL80^s proteins. Given that LAC9 is responsive to the GAL80 protein, we predicted that it would respond to GAL80^s proteins. The ability of GAL4 and LAC9 to activate the *MEL1* gene under both noninducing and inducing conditions was assayed in the presence of either *GAL80* or *GAL80*^s alleles borne on single-copy plasmids (Table 2). Although the wild-type *GAL80* allele did not cause repression of LAC9 under noninducing conditions, all three *GAL80*^s alleles did. By contrast, both *GAL80* and *GAL80*^s alleles caused repression of GAL4 under noninducing conditions. The *GAL4* gene elicited an uninducible phenotype in combination with all three *GAL80*^s alleles. The *LAC9* gene also elicited an uninducible phenotype in combination with the *GAL80*^{s-0} and *GAL80*^{s-2} alleles; however, in combination with the *GAL80*^{s-1} allele, an inducible phenotype similar to that produced by the wild-type *GAL4* and *GAL80* genes resulted. We concluded that the LAC9 protein

TABLE 2. Effect of *GAL80*^s alleles on LAC9-activated transcription

Activator protein and <i>GAL80</i> allele ^a	Mean relative α -galactosidase level \pm SE under indicated conditions		R ^b
	Uninduced	Induced	
LAC9			
<i>gal80</i>	66.7 \pm 8.5	69.2 \pm 3.0	1.0
<i>GAL80</i>	63.7 \pm 9.4	142.3 \pm 23.9	2.2
<i>GAL80</i> ^{s-0}	17.5 \pm 3.8	23.5 \pm 6.4	1.3
<i>GAL80</i> ^{s-1}	13.7 \pm 1.3	53.8 \pm 12.8	3.9
<i>GAL80</i> ^{s-2}	13.2 \pm 1.7	8.5 \pm 2.1	0.6
GAL4			
<i>gal80</i>	83.8 \pm 29.9	119.2 \pm 25.6	1.4
<i>GAL80</i>	12.5 \pm 0.4 ^c	100.0 \pm 0.8 ^d	8.0
<i>GAL80</i> ^{s-0}	12.8 \pm 1.3	10.7 \pm 3.8	0.8
<i>GAL80</i> ^{s-1}	10.3 \pm 0.8	17.5 \pm 4.3	1.7
<i>GAL80</i> ^{s-2}	12.4 \pm 2.1	12.0 \pm 7.3	1.0

^a Activator proteins were encoded by either pSB32-LAC9 or pSB32-GAL4 in strain YJ0. *GAL80* alleles were borne on YCp50 in strain YJ0.

^b R, Induction ratio for α -galactosidase (induced level to uninduced level).

^c The relatively high level of α -galactosidase activated under noninducing conditions (cf. Table 1) is characteristic of strains in which the *GAL80* gene is plasmid borne.

^d The reference value 100.0 represents an increase in optical density at 400 nm of 0.333/min per 10⁷ cells.

interacts with the GAL80^s proteins and that it does so in either an inducible or uninducible manner, depending on the particular GAL80^s protein involved.

The GAL4 and LAC9 carboxyl termini are functionally identical. Our results indicate that the GAL80 protein is able to repress LAC9 activity in *S. cerevisiae*, but in contrast to repression of GAL4, repression of LAC9 takes place only when GAL80 is overproduced. Exploring the reason for the differential responses of LAC9 and GAL4 to GAL80 could yield new information about the mechanism of action of GAL80 and GAL80^s proteins and would clarify further the similarities and differences between the *S. cerevisiae* and *K. lactis* galactose regulons. Two explanations for the difference in the responses of LAC9 and GAL4 to GAL80 seemed likely, based on what is known of the requirements for proper regulation of GAL4 by GAL80 (described in the introduction). First, amino acid differences in LAC9 relative to GAL4 might result in a weak interaction between LAC9 and GAL80 molecules such that an excess of GAL80 would be required to effect repression of LAC9. Second, the amount of LAC9 protein produced from a single copy of the *LAC9* gene in *S. cerevisiae* might exceed the amount of GAL4 protein produced from a single copy of the *GAL4* gene. In the latter case, the steady-state level of LAC9 protein in vivo might be high enough to titrate the GAL80 protein present, which would allow for constitutive expression of galactose regulon structural genes.

We first tested the possibility that amino acid sequence differences between LAC9 and GAL4 in the carboxy-terminal region of similarity, the region in GAL4 at which GAL80 interacts (20, 28), cause the difference in the responses of LAC9 and GAL4 to GAL80. If we could show that any of these amino acid differences caused LAC9 to escape repression, the implication would be that the corresponding amino acids of GAL4 are critical for proper interaction with GAL80. Within this region, there are three differences between the LAC9 and GAL4 protein sequences. (i) LAC9 contains an Ile residue at a position where GAL4

activator protein	relative α -galactosidase level	
	uninduced	induced
1. GAL4-FNTTMDVVYNY L FDDE DT PPNP K KE (wt)	1.0	100.0
2. GAL4-FNTTMDVVYNY L FDDE DT PPNP K KE	0.5	101.1
3. GAL4-FNTTMDVVYNY L FD N DE DT PPNP K KE	0.6	108.4
4. GAL4-FNTTMDVVYNY L FD N DE DT PPNP K KE	1.0	86.2
5. GAL4-FNTTMDVVYNY L FDDE	0.8	99.1
6. GAL4-FNTTMDVVYNY L FDDE	0.2	113.4
7. GAL4-FNTTMDVVYNY L FD N DE	1.5	118.8
8. GAL4-FNTTMDVVYNY L FD N DE	0.3	114.9
9. LAC9-FNTTMDVVYNY L FD N DE (wt)	84.0	67.9
10. LAC9-FNTTMDVVYNY L FDDE	85.3	106.0
11. LAC9-FNTTMDVVYNY L FDDE	64.9	89.2

FIG. 1. α -Galactosidase enzyme activities activated by GAL4 and LAC9 carboxy-terminal variants. The amino acid sequence after residue 855 in each GAL4 variant, and residue 847 in each LAC9 variant, is shown. Amino acids unique to wild-type GAL4 are in boldface; amino acids unique to wild-type LAC9 are italicized. Asterisks indicate single amino acid alterations introduced into the GAL4 and LAC9 proteins. The genes encoding the variant proteins were borne on YCp50 in strain YJ4. The α -galactosidase enzyme activity level activated by wild-type GAL4 under inducing conditions has been set to 100.0 and represents an increase in optical density at 400 nm of 0.916/min per mg of protein in cell extracts.

contains Leu (Fig. 1, lines 1 and 9). (ii) LAC9 contains an Asn residue three residues downstream of this position as an insertion relative to GAL4. (iii) LAC9 terminates after the final acidic amino acid in this region, Glu, while the GAL4 sequence continues for another nine amino acids. To test whether these sequence differences are responsible for the difference in the ability of GAL80 to interact with LAC9 and GAL4, we constructed an array of mutant *GAL4* alleles that encode variant GAL4 proteins which represent all permutations of hybrid GAL4-LAC9 carboxyl termini (Fig. 1, lines 2 through 8). We also constructed two mutant *LAC9* alleles that encode variants that contained features of the GAL4 carboxyl terminus (Fig. 1, lines 10 and 11). All the variant activator proteins were tested for the ability to interact with GAL80 by measuring the amount of α -galactosidase activity they activated under noninducing and inducing conditions in the presence of wild-type amounts of GAL80. All GAL4 variants (Fig. 1, lines 2 through 8) activate α -galactosidase activity to high levels under inducing conditions and low levels under noninducing conditions in a manner similar to that of wild-type GAL4 (Fig. 1, line 1). All LAC9 variants (Fig. 1, lines 10 and 11) activate α -galactosidase activity to high levels under both inducing and noninducing conditions in a manner similar to that of wild-type LAC9 (Fig. 1, line 9). These results indicate that sequence differences in the carboxy-terminal region of similarity have, at the most, minor effects on the ability of GAL80 to interact with LAC9 and GAL4.

LAC9-containing yeast strains possess high levels of activator protein. Given these results, we investigated the alternate possibility that the LAC9 protein exists at a higher level than GAL4 does in *S. cerevisiae*, which would lead to titration of GAL80 and constitutive structural gene activation. Two obvious methods for protein quantitation, Western blotting

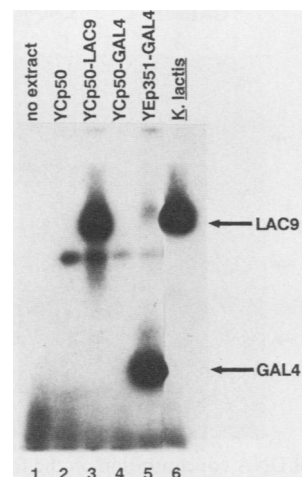


FIG. 2. Electrophoretic mobility shift assay of UAS_G3-4 with 100 μ g of total cellular protein extract from either *S. cerevisiae* YJ4 transformed with the plasmids indicated (lanes 1 through 5) or *K. lactis* Y-1140 (lane 6), and 0.8 ng of [³²P]UAS_G3-4.

(immunoblotting) and assaying the activities of *GAL4-lacZ* and *LAC9-lacZ* fusion genes, were not feasible because these methods cannot detect the low amounts of the LAC9 and GAL4 proteins produced from single-copy plasmids (20, 21, 24, 36, 41). Although the GAL4 protein can be detected on Western blots when overproduced (20, 21), overproduction of LAC9 and GAL4 might not reflect the relative levels under wild-type conditions, the conditions under which LAC9 and GAL4 differ in response to GAL80. Therefore, to quantify the relative steady-state levels of LAC9 and GAL4 in *S. cerevisiae*, we utilized an electrophoretic mobility shift assay that detects these proteins by their ability to bind a GAL4 binding site (termed UAS_G3-4; described in Materials and Methods). The assay is based on the fact that a protein-UAS_G3-4 complex exhibits reduced mobility in an agarose gel, relative to free UAS_G3-4. The presence of the GAL4 and LAC9 proteins in these complexes has been confirmed experimentally by using antibody to GAL4 protein and DNAs containing different GAL4 and LAC9 binding sites (S. D. Langdon and S. A. Johnston, unpublished results). There are certain limitations to the use of this assay in quantifying the relative levels of LAC9 and GAL4. The assay must be performed under conditions of saturating DNA to control for possible differences in affinity for UAS_G3-4 and to assure that all the LAC9 and GAL4 protein in each extract is detected. It must be demonstrated that the LAC9-UAS_G and GAL4-UAS_G complexes have approximately equal stabilities. It must be assumed that the two proteins bind DNA in a similar manner and are equally extractable from whole cells.

Despite these limitations, we felt that this assay would give us the best estimation of the relative steady-state levels of LAC9 and GAL4 produced from single-copy genes. When UAS_G3-4 (run alone in Fig. 2, lane 1) was incubated with an extract from a *gal4Δ* *S. cerevisiae* strain, a single complex containing neither LAC9 nor GAL4 was observed (Fig. 2, lane 2). When UAS_G3-4 was incubated with extracts from either *S. cerevisiae* (Fig. 2, lane 3) or *K. lactis* (Fig. 2, lane 6) strains containing single copies of the *LAC9* gene, a second complex was evident, representing the binding of UAS_G3-4 by the LAC9 protein. When UAS_G3-4 was incubated with an extract from a *S. cerevisiae* strain containing a

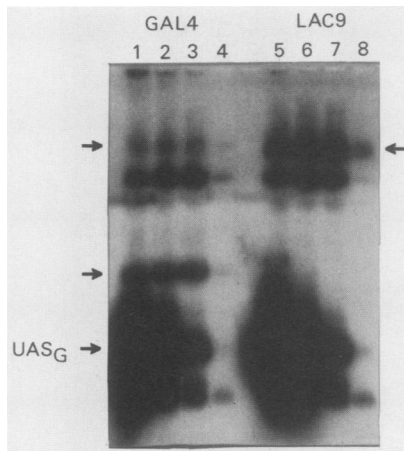


FIG. 3. Effect of DNA concentration on the formation of GAL4-UAS_G and LAC9-UAS_G complexes. Electrophoretic mobility shift assays were performed with 50 μ g of extract from YJ4 transformed with YEp351-GAL4 (lanes 1 through 4) YEp50-LAC9 (lanes 5 through 8) and 20 (lanes 1 and 5), 4 (lanes 2 and 6), 0.8 (lanes 3 and 7), or 0.08 (lanes 4 and 8) ng of [³²P]UAS_G3-4. Unlabeled arrows denote the GAL4-UAS_G complexes (left) and the LAC9-UAS_G complex (right).

single copy of the *GAL4* gene, however (Fig. 2, lane 4), no additional binding to UAS_G3-4 above background (Fig. 2, lane 2) was observed. This was not caused by an overall inability to detect binding of the GAL4 protein to UAS_G3-4, since an extract from a strain containing the *GAL4* gene on the multicopy plasmid YEp351 (15) has significant amounts of UAS_G3-4 binding activity (Fig. 2, lane 5), and overexposure of the autoradiograph revealed a faint band in Fig. 2 comigrating with the GAL4-UAS_G3-4 complex indicated (Fig. 2, lane 5). We conclude that the extracts from both LAC9-containing strains demonstrate higher amounts of activator protein per unit of total cellular protein than does the extract from the single-copy *GAL4* strain.

To confirm that the assay described in Fig. 2 was performed under conditions of saturating DNA, it was repeated with the same amounts of protein and increasing amounts of DNA per lane. If some of the GAL4 or LAC9 protein remained uncomplexed with DNA and undetected in Fig. 2, additional complex formation should have been evident after the amount of UAS_G3-4 was increased. Figure 3 shows that increasing the amount of DNA per reaction fivefold (lanes 2 and 6) or 25-fold (lanes 1 and 5) over that indicated in Fig. 2 (lanes 3 and 7) did not lead to an increase in the amount of complexed UAS_G3-4. We conclude that the complexes observed in Fig. 2 represent most of the functional LAC9 and GAL4 present in the cell extracts.

The LAC9-UAS_G and GAL4-UAS_G complexes have approximately equal stabilities. We also allowed for the possibility that the LAC9-UAS_G3-4 and GAL4-UAS_G3-4 complexes have different stabilities. We added equal amounts of LAC9 and GAL4 DNA-binding activity to [³²P]UAS_G3-4 (Fig. 4, lanes 1 and 9) and then added a fivefold excess of unlabeled UAS_G3-4 to each binding reaction. At specific time points, portions of the protein-DNA mixture were removed for analysis on an agarose gel. If the GAL4-UAS_G3-4 complex is less stable than the LAC9-UAS_G3-4 complex, the [³²P]UAS_G3-4 should be replaced by unlabeled UAS_G3-4 at a faster rate when bound by GAL4 than when bound by LAC9. The result will be that the GAL4-[³²P]UAS_G3-4 complex will have a shorter half-life than that of the LAC9-

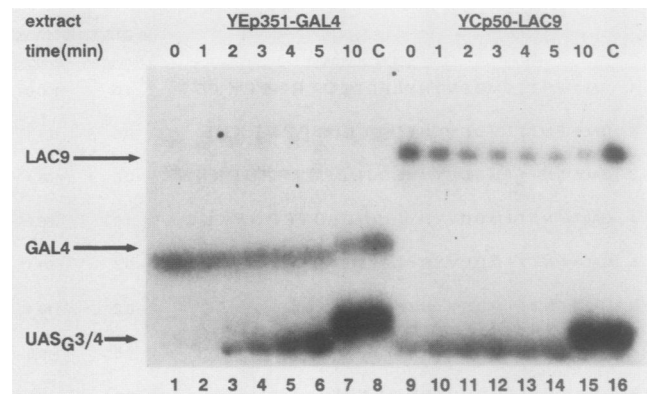


FIG. 4. Dissociation of GAL4 and LAC9 proteins from UAS_G3-4. Bands represent the reaction products of the binding of 25 μ g of GAL4-containing (lanes 1 through 8) or LAC9-containing (lanes 9 through 16) *S. cerevisiae* extract with 0.1 ng of [³²P]UAS_G3-4, followed by the addition of 0.5 ng of unlabeled UAS_G3-4 and incubation for the specified time. C, Controls, in which protein-DNA complexes were not exposed to unlabeled UAS_G3-4.

[³²P]UAS_G3-4 complex. A comparison of lanes 15 and 16 and lanes 7 and 8 of Fig. 4 shows that after a 10-min exposure to competitor, most of the LAC9 protein had dissociated from the [³²P]UAS_G3-4, whereas the same or perhaps a smaller amount of GAL4 had dissociated, which indicates that LAC9 dissociated from [³²P]UAS_G3-4 at a rate at least as fast as GAL4. We conclude that the stability of the LAC9-UAS_G3-4 complex is no greater than the stability of the GAL4-UAS_G3-4 complex and that the apparent differences in the amounts of LAC9 and GAL4 proteins per cell indicated by Fig. 2 cannot be explained by differences in the stabilities of the two protein-DNA complexes.

DISCUSSION

Our studies show that the *S. cerevisiae* GAL80 protein is able to recognize and repress the *K. lactis* LAC9 activator protein, the homolog of GAL4 from milk yeast (Table 1). Presumably, the LAC9 and GAL4 proteins are evolutionarily related, having diverged from a common ancestral *trans*-acting factor. It is, therefore, not surprising to find that at least some features of the GAL4 protein that are recognized by GAL80 are present in LAC9. Almost certainly, these features include the highly conserved carboxy-terminal region known to possess GAL80-interactive function in GAL4 (20, 28). We have shown that the sequence differences between the two activator proteins in this region have little, if any, effect on the ability of GAL80 to interact with LAC9 and GAL4 (Fig. 1), which indicates that the carboxy-terminal nine amino acids and the presence of a Leu residue at position 868 in GAL4 are not required for GAL4-GAL80 interaction. We presume, therefore, that GAL80 effects repression of LAC9 through the LAC9 carboxyl terminus, although additional experiments are required to prove this.

Although the amino acids at the LAC9 carboxyl terminus permit interaction with GAL80, LAC9 escapes repression *in vivo*. A comparison of the amounts of DNA bound in electrophoretic mobility shift assays (Fig. 2, lanes 3 and 4) suggests that LAC9 exists at a much higher level in *S. cerevisiae* than does GAL4. Overexpression of the *GAL4* gene on the multicopy plasmid YEp351 raises the intracellular GAL4 protein level to one approximating that of LAC9 (Fig. 2, lanes 3 and 5). Since YEp plasmids normally exist at

roughly 50 copies per cell (6), 50-fold may be taken as an estimate of the difference in the amounts of LAC9 and GAL4 present under wild-type conditions in *S. cerevisiae*. Since raising the level of GAL4 protein five- to 10-fold causes constitutive regulon expression (19), it is likely that LAC9 escapes repression by GAL80 because it exists at a high level in vivo. Supporting this idea is the observation that increasing the intracellular level of GAL80 elicits repression of LAC9 (Table 1). We have not excluded the possibility that sequence differences between LAC9 and GAL4 in a region outside the carboxyl termini determine in part the difference in interaction with GAL80. However, since all six sequenced *GAL4^c* mutations affect the GAL4 carboxyl terminus (20; Salmeron and Johnston, submitted), GAL80 probably interacts with GAL4 only at this region. Additionally, the fact that the GAL80^s proteins repress LAC9 at normal levels suggests that the conformation of LAC9 does not interfere sterically with the binding of GAL80.

While overproduction of the GAL80 protein is required to mediate repression of LAC9, normal levels of GAL80^s proteins repress LAC9 (Table 2). This observation leads to two possible implications for the nature of the GAL80^s proteins. First, GAL80^s proteins may exist at higher levels in vivo than GAL80 does. We consider this unlikely, since the *GAL80^s* mutations are point mutations within the *GAL80* coding sequence (31), not within the *GAL80* promoter as would be expected if they led to overexpression of the wild-type *GAL80* gene. Furthermore, the mutant alleles all encode GAL80 variants with altered charge, a situation more likely to induce destabilization than stabilization. The second possibility is that the *GAL80^s* alleles encode proteins with higher affinity for both LAC9 and GAL4. Certainly, our results argue against the idea that the *GAL80^s* mutations simply affect the inducer-binding function of GAL80. If this was the case, with no concomitant increase in ability to bind LAC9, then LAC9 would escape repression mediated by the GAL80 and GAL80^s proteins equally well, through its high dosage. The inducible nature of the LAC9-GAL80^{s-1} interaction also argues against a loss of inducer-binding function, at least for this GAL80^s protein. Therefore, we believe that the *GAL80^s* mutations we examined increase the affinity of GAL80 for both LAC9 and GAL4 proteins.

Our results lead us to propose that the LAC9, GAL80, and GAL80^s proteins exist in *S. cerevisiae* at levels much higher than GAL4. The LAC9 level is high enough to allow escape from GAL80-repression under noninducing conditions, while GAL4 function is repressed. *GAL80^s* mutations increase the affinity of GAL80 for both GAL4 and LAC9, resulting in repression of LAC9 under noninducing conditions. The effect of inducer is to alter in some way the interaction between all LAC9/GAL4 and GAL80/GAL80^s protein pairs. In some cases, i.e., the LAC9-GAL80^{s-1} and GAL4-GAL80 interactions, this alteration is sufficient to relieve the activator protein from repression. However, in many cases it is not, which would result in an uninducible phenotype.

The carboxy-terminal activation regions of LAC9 and GAL4 show nearly complete sequence identity (36). Since, for the most part, the two protein sequences are completely dissimilar (36), sequence conservation in this region must reflect a functional constraint. The primary sequence requirements for activation regions in yeast cell positive regulatory proteins are minimal (11, 29), so it is unlikely that the activation function has constrained evolution of the LAC9 and GAL4 carboxyl termini. Given our results and the fact that a homolog to GAL80, termed LAC10, exists in *K. lactis*

(8), it is more likely that the high degree of carboxy-terminal sequence similarity results from the interaction of LAC9 and GAL4 with a negative regulatory protein, an interaction that may impart stringent primary sequence requirements on both positive and negative elements. Therefore, we predict that a region of similarity may exist between the GAL80 and LAC10 proteins that is likely to constitute the region that functions in interaction with GAL4 and LAC9.

Our electrophoretic mobility shift assay demonstrated equal amounts of DNA-binding activity in LAC9-containing extracts from *S. cerevisiae* and *K. lactis* (Fig. 2, lanes 3 and 6). This implies that *K. lactis* cells possess the same level of LAC9 protein as do *S. cerevisiae* cells. A high level of LAC9 in *K. lactis* could explain two phenomenological differences between the galactose metabolism regulatory systems of *K. lactis* and *S. cerevisiae*. First, *K. lactis* structural genes are expressed at significant levels in the absence of inducer (33, 41), while *S. cerevisiae* genes are either nearly or completely repressed. Second, glucose has little or no effect on the level of activation of the *K. lactis* regulon in most strains (7), whereas glucose severely represses expression of the *S. cerevisiae* regulon (1). Both *K. lactis* traits can be mimicked in *S. cerevisiae* by overexpression of the *GAL4* gene (19) or by replacing the *GAL4* gene with the *LAC9* gene (41). Similarly, replacement of the *LAC9* gene in *K. lactis* with the *GAL4* gene causes glucose repression of the *K. lactis* regulon (34). Together, these results suggest that a high level of LAC9 protein in *K. lactis*, relative to the level of GAL4 in *S. cerevisiae*, may be partially responsible for the *K. lactis* pattern of gene expression. Although more must be learned about the *K. lactis* regulatory system before any models can be proven, further comparative studies between the components of the galactose metabolism regulons of *K. lactis* and *S. cerevisiae* should prove fruitful in elucidating the mechanism and evolution of this regulatory system in both yeast species.

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